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14. ABSTRACT The ability of Trastuzumab, a drug for treatment of HER2 overexpressing breast cancer, to induce apoptosis in HER2 overexpressing breast cancer lines was investigated. Particular attention was paid to the role of Akt downregulation by Trastuzumab and the resulting upregulation of pro-apoptotic Akt substrates. Trastuzumab was found to induce very little apoptosis, and a few Akt substrates investigated, Bad and FKHR, were not affected by Trastuzumab treatment. Additional studies investigated the affect of Trastuzumab on the upregulation of the GLUT1 glucose transporter in HER2 overexpressing breast cancer cells. Trastuzumab inhibited the Cobalt-dependent induction of GLUT1. This indicates Trastuzumab may have affects on hypoxic tumors because Cobalt stimulation mimics a cell's response to hypoxia which results in induction of GLUT1 which in turn accelerates aerobic or anaerobic glycolysis for tumor growth and survival.					
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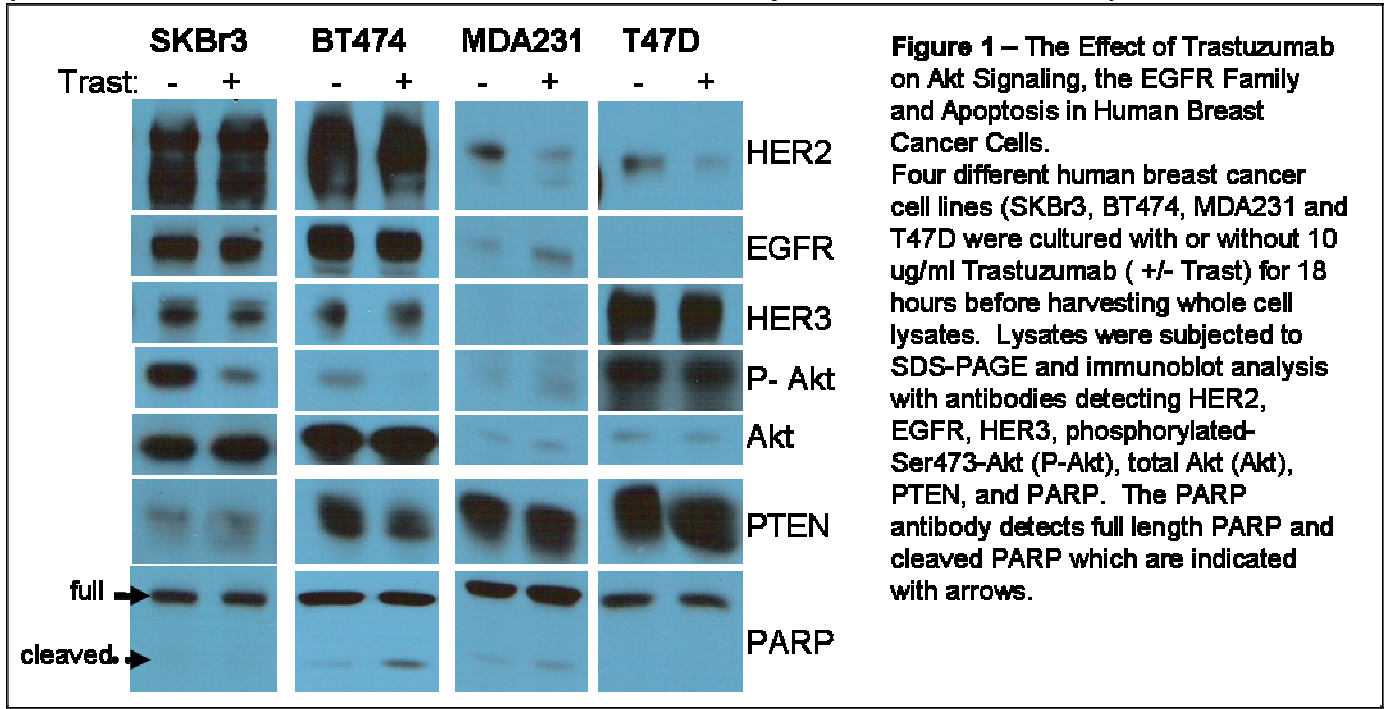
INTRODUCTION

Trastuzumab is a drug which specifically targets HER2, an oncogene product overexpressed in a subset of breast cancers (1). Trastuzumab has been hailed for its specificity in targeting HER2 overexpressing cancer cells in contrast to standard chemotherapy regimens which target rapidly dividing cells and cause major side effects in treated patients. However, Trastuzumab only works in a third of patients with HER2 overexpressing breast cancer and has mild adverse side effects in 40% of patients and major adverse side effects in 5% of patients (2). Therefore, fully understanding the biology of Trastuzumab is important in order to identify patients who will respond to Trastuzumab therapy and to design combinations of therapies that will result in more patients responding. The proposed study seeks to understand one of the signaling pathways affected by cells treated with Trastuzumab: the Akt pathway, which is known to mediate cell survival (3-5). Trastuzumab has been shown to cause cell cycle arrest in HER2 overexpressing cells (6) and sometimes induce apoptosis (6, 7). However, HER2 overexpressing cells can become resistant to Trastuzumab if Akt is not downregulated (8, 9). The approved study seeks to understand whether downregulation of Akt by Trastuzumab induces apoptosis, and if so, which molecules downstream of Akt action are necessary targets of Trastuzumab action.

BODY

Aim 1: The first aim of this proposal was to assess the effectiveness of Trastuzumab at inducing cell death in cultured breast cancer cells which overexpress HER2. Secondly, this aim sought to address whether expression of constitutively active Myr-Akt1 would lead to Trastuzumab resistance.

Two human breast cancer cell lines, SKBr3 and BT474, both of which overexpress HER2 and are sensitive to Trastuzumab (9) were obtained from the ATCC. These cell lines were compared to two other human breast cancer cell lines which do not overexpress HER2: MDA231 and T47D. All cell lines were maintained in complete media (DMEM containing 10% FBS, 0.1 mM non-essential amino acids and 40 ng/ml insulin). Parallel cell monolayers were grown to near confluence and one dish was treated with 10 µg/ml Trastuzumab in complete media while the other was left untreated. Cells were incubated overnight and cell lysates were harvested 18 hours after treatment and then subjected to immunoblot analysis.



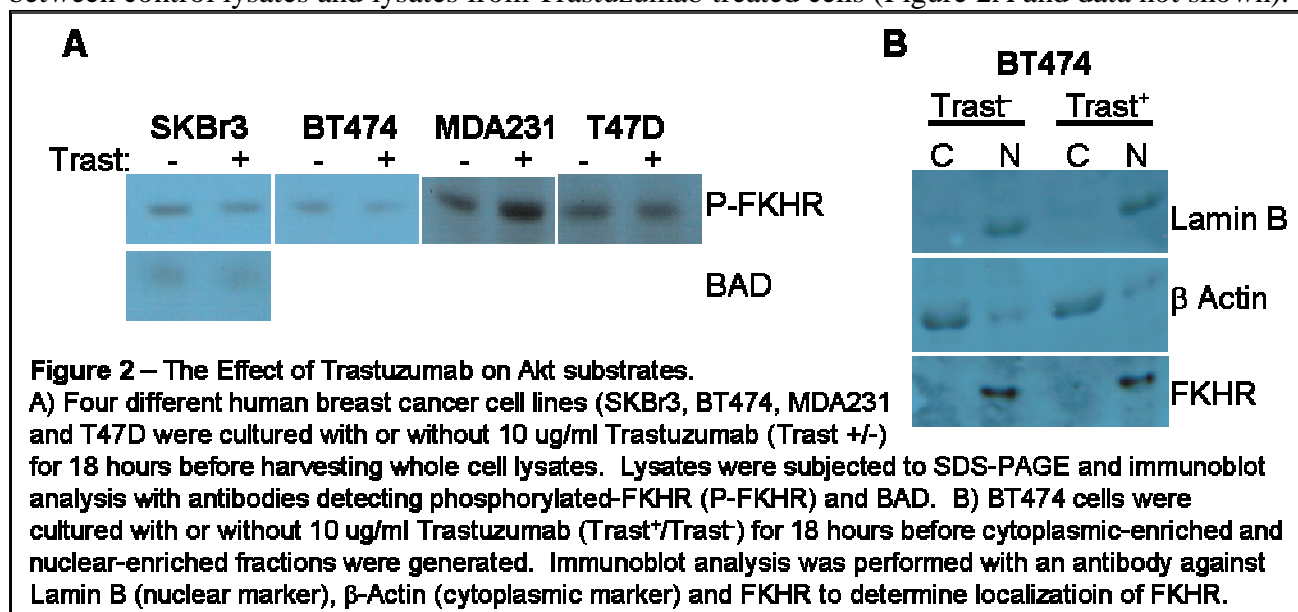
Previous reports have shown downregulation of HER2 after Trastuzumab treatment (10), but this has been disputed (6, 11). Our data indicates Trastuzumab does not regulate HER2 in the two cell lines which clearly overexpress HER2: SKBr3 and BT474 (Figure 1). MDA231 and T47D do not overexpress HER2 (Figure 1) and do not respond to trastuzumab. The decrease in HER2 expression after trastuzumab treatment in MDA231 and T47D cells (Figure 1) is likely inconsequential because HER2 is not overexpressed and HER2 is not responsible for tumorigenesis in these cell lines. EGFR and HER3, two other members of the EGFR family

were also examined, and were also unaffected by Trastuzumab treatment. Trastuzumab does inhibit activation of Akt as seen by a decrease in Akt phosphorylation at Serine 473 after trastuzumab treatment in the two HER2 overexpressing cell lines, but not in the other two cell lines (Figure 1). Total levels of Akt and PTEN, a negative regulator of Akt activation, are not affected by trastuzumab treatment (Figure 1). This supports the hypothesis that downregulation of Akt by Trastuzumab in HER2 overexpressing cells could lead to activation of pro-apoptotic molecules normally regulated by Akt. Apoptosis was examined by probing for PARP, a substrate of caspases and thus a marker of apoptosis. BT474 cells demonstrate an increase in apoptosis (marked by increased level of cleaved PARP) after trastuzumab treatment, but trastuzumab treatment does not lead to apoptosis in SKBr3 cells (Figure 1). Thus, downregulation of Akt activation by trastuzumab in SKBr3 cells is not sufficient to cause apoptosis, but trastuzumab does cause an increase in apoptosis in BT474 cells.

The lack of induction of apoptosis in SKBr3 cells and the modest induction of apoptosis in BT474 cells after Trastuzumab treatment indicate that apoptosis may not be a major effect of Trastuzumab, at least in cultured cell. Perhaps Trastuzumab induction of cell cycle arrest is more important to its anti-tumor effects (11) than induction of apoptosis. Alternatively, apoptosis may occur *in vivo*, in the context of an immune system and a tumor microenvironment, but not in cell culture. The second part of this aim sought to examine whether constitutively active Myr-Akt1 could rescue Trastuzumab induction of apoptosis, but this was not examined since Trastuzumab failed to induce apoptosis robustly. Other groups have demonstrated that activation of Akt causes Trastuzumab resistance (11, 12), but this is likely via affects other than apoptosis.

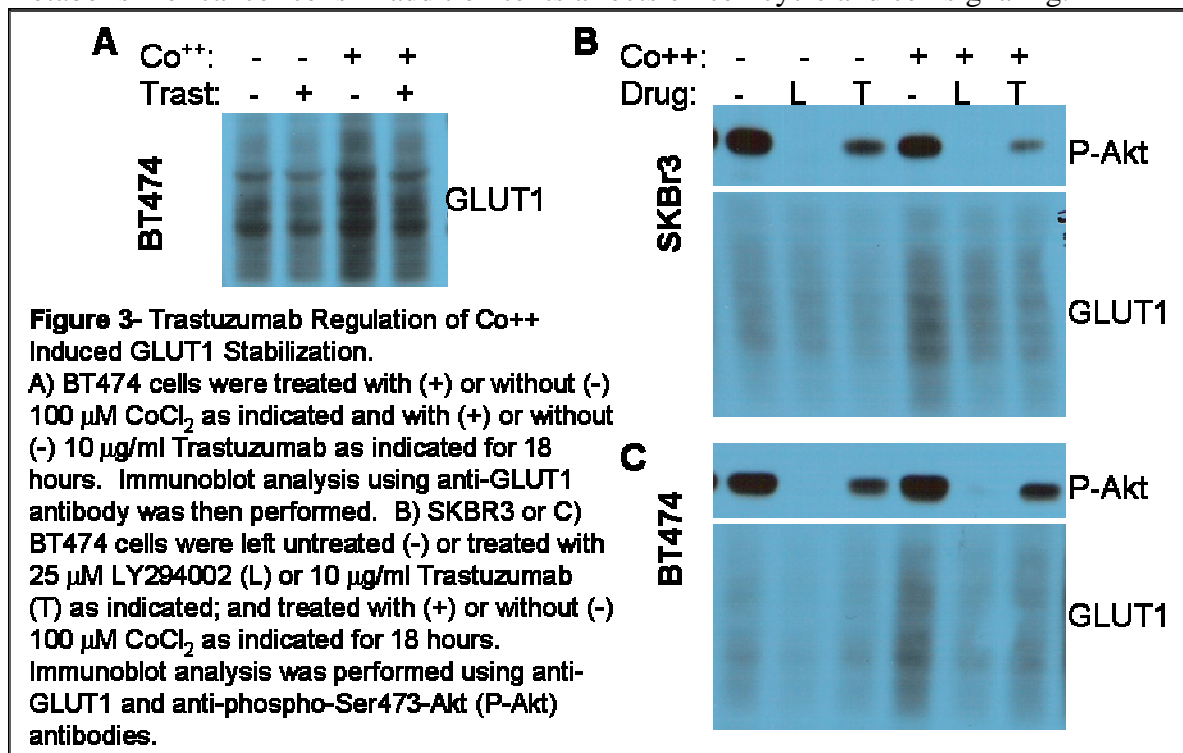
Aim 2: The second aim of this proposal was to determine substrates downstream of Akt critical to Trastuzumab-induced apoptosis.

Lysates of cells treated 18 hours with 10 μ g/ml Trastuzumab or lysates from untreated cells were used to examine the status of a few pro-apoptotic Akt substrates. FKHR is a transcription factor which is responsible for transcription of genes involved in cell cycle arrest and apoptosis (4). Akt phosphorylation of FKHR causes its transport out of the nucleus which attenuates transcription of pro-apoptotic and cell cycle arrest genes. Therefore, the phosphorylation status of FKHR was examined by immunoblot using a phospho-specific antibody which recognizes FKHR which has been phosphorylated by Akt. Trastuzumab down-regulation of Akt would be expected to cause a decrease in FKHR phosphorylation if FKHR is indeed a substrate in HER2 overexpressing breast cancer cells. However, Trastuzumab caused no difference in the phosphorylation of FKHR (Figure 2A). FKHR subcellular localization in BT474 cells was also examined before and after trastuzumab treatment, but FKHR appeared to be mostly nuclear (in the same fraction enriched for Lamin B), regardless of treatment (Figure 2B). Bad is another pro-apoptotic substrate of Akt and is eventually degraded after phosphorylation by Akt (3). Bad was also evaluated by immunoblot, but no differences were detected between control lysates and lysates from Trastuzumab treated cells (Figure 2A and data not shown).



Trastuzumab was not efficiently inducing apoptosis in HER2 overexpressing breast cancer cells and the few pro-apoptotic substrates of Akt which were examined were also not being affected by Trastuzumab. Therefore, studies of Trastuzumab induction of apoptosis and further identification of pro-apoptotic Akt substrates downstream of Trastuzumab treatment were halted. However, identification of other important downstream affects of Akt inactivation following Trastuzumab treatment were pursued.

Akt has been shown to induce aerobic glycolysis in models of hematopoietic malignancies, partly via activation of the GLUT1 glucose transporter (13, 14). Additionally, it has been shown that HER2 overexpressing cancers stabilize HIF (hypoxia inducible factor) even in the absence of hypoxia, and HIF activates GLUT1 transcription (15, 16). Therefore, we wanted to test whether Trastuzumab had an affect on GLUT1 expression in both normoxic conditions and in the CoCl_2 model of hypoxia which stabilizes the HIF complex similar to hypoxia. BT474 were left untreated, treated with 100 μM CoCl_2 , treated with 10 $\mu\text{g/ml}$ Trastuzumab, or treated with both 100 μM CoCl_2 and 10 $\mu\text{g/ml}$ Trastuzumab and incubated overnight. CoCl_2 clearly activated GLUT1 expression (Figure 3A, compare lane 1 to lane 3) while Trastuzumab alone slightly decreased GLUT1 expression (Figure 3A, compare lane 1 to lane 2). Additionally, Trastuzumab attenuated the CoCl_2 activation of GLUT1 (Figure 3A, compare lane 3 to lane 4). To investigate whether the trastuzumab affect on GLUT1 expression could be though Akt, a PI3K inhibitor, LY294002 (LY), was used to investigate whether inhibition of PI3K and Akt would recapitulate the results observed with Trastuzumab. SKBr3 and BT474 cells were left untreated or treated with 25 μM LY, 10 $\mu\text{g/ml}$ Trastuzumab, 100 μM CoCl_2 , both 25 μM LY and 100 μM CoCl_2 , or both 10 $\mu\text{g/ml}$ Trastuzumab and 100 μM CoCl_2 and incubated overnight. In both cell types, Akt was inactivated completely by LY and was partially inhibited by Trastuzumab while CoCl_2 treatment alone had no effect on Akt (Figure 3B and 3C). In SKBr3 cells, Trastuzumab and LY had no effect on basal GLUT1 levels, but both drugs inhibited the increase in GLUT1 seen after CoCl_2 treatment (Figure 3B). The inhibition was not complete because GLUT1 levels are still higher in drug plus CoCl_2 treated samples than the basal level seen without CoCl_2 . This suggests that the increase in GLUT1 after CoCl_2 treatment requires PI3K or Akt, which are inhibited by LY and Trastuzumab. In BT474 cells, treatment with LY reduced the basal level of GLUT1 and also inhibited the CoCl_2 activation of GLUT1 (Figure 3C). Trastuzumab had a similar, but less pronounced affect as LY. The observation that LY strongly inhibits Akt activation and also strongly reduces GLUT1 (both with and without CoCl_2 treatment) and that Trastuzumab moderately inhibits Akt activation and moderately reduces GLUT1 activation by CoCl_2 suggests that Trastuzumab inactivation of Akt may be responsible for the decreased level of GLUT1. These results also indicate Trastuzumab may affect the metabolism of cancer cells in addition to its affects on cell cycle and cell signaling.



KEY RESEARCH ACCOMPLISHMENTS

- Trastuzumab inhibits Akt activity in HER2 overexpressing breast cancer cell lines
- Inhibition of Akt activity by Trastuzumab fails to upregulate two pro-apoptotic substrates of Akt: FKHR and Bad
- Trastuzumab does not robustly induce apoptosis in vitro
- Trastuzumab inhibits the expression of GLUT1 and cobalt-induced increase in GLUT1 expression in a manner similar to inhibition of PI3K/Akt
- Expression of EGFR family members, including HER2, is the same before and after Trastuzumab treatment

REPORTABLE OUTCOMES

- This work has been presented at the UCHSC Pathology Department Research in Progress Seminar

CONCLUSIONS

Using two HER2 overexpressing human breast cancer cell lines, SKBr3 and BT474, we demonstrated inactivation of Akt by Trastuzumab. However, inactivation of Akt failed to induce apoptosis in these cell lines. In agreement, Trastuzumab failed to upregulate two important pro-apoptotic substrates of Akt, Bad and FKHR. Additionally, the expression of HER2 and the other receptors of the EGFR family was similar before and after Trastuzumab treatment. Trastuzumab decreased the expression of the GLUT1 glucose transporter and also inhibited its cobalt-dependent increase in expression similar to inhibition of the PI3K/Akt pathway. This suggests the inhibition of Akt by Trastuzumab may be responsible for the reduced GLUT1 expression. The subcellular localization of GLUT1 before and after Trastuzumab treatment remains to be determined, but would be interesting to note because plasma membrane localization of GLUT1 is an important route for glucose import in cancer cells which perform excessive glycolysis. Thus, while Trastuzumab does not seem to induce apoptosis, it may effect the metabolic needs of cancer cells via down regulation of GLUT1 and it does inhibit activation of Akt which others have shown causes growth arrest and sensitizes cancer cells to other drugs (11, 12). Continued research to better understand how Trastuzumab functions and how it can best be used to treat breast cancer with maximum efficacy and minimum side effects is an important and currently active area of research.

REFERENCES

- 1 Menard,S., Pupa,S.M., Campiglio,M. and Tagliabue,E. Biologic and therapeutic role of HER2 in cancer, *Oncogene*, 22: 6570-6578, 2003.
- 2 Vogel,C.L., Cobleigh,M.A., Tripathy,D., Gutheil,J.C., Harris,L.N., Fehrenbacher,L., Slamon,D.J., Murphy,M., Novotny,W.F., Burchmore,M., Shak,S., Stewart,S.J. and Press,M. Efficacy and Safety of Trastuzumab as a Single Agent in First-Line Treatment of HER2-Overexpressing Metastatic Breast Cancer, *J Clin Oncol*, 20: 719-726, 2002.
- 3 Del Peso,L., Gonzalez-Garcia,M., Page,C., Herrera,R. and Nunez,G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt, *Science*, 278: 687-689, 1997.
- 4 Burgering,B.M.T. and Medema,R.H. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty, *J Leukoc Biol*, 73: 689-701, 2003.
- 5 Gottlieb,T.M., Leal,J.F.M., Seger,R., Taya,Y. and Oren,M. Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis, *Oncogene*, 21: 1299-1303, 2002.
- 6 Sliwkowski,M.X., Lofgren,J.A., Lewis,G.D., Hotaling,T.E., Fendly,B.M. and Fox,J.A. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin), *Semin.Oncol.*, 26: 60-70, 1999.

- 7 Mohsin,S.K., Weiss,H.L., Gutierrez,M.C., Chamness,G.C., Schiff,R., Digiovanna,M.P., Wang,C.X., Hilsenbeck,S.G., Osborne,C.K., Allred,D.C., Elledge,R. and Chang,J.C. Neoadjuvant trastuzumab induces apoptosis in primary breast cancers, *J Clin.Oncol.*, 23: 2460-2468, 2005.
- 8 Chan,C.T., Metz,M.Z. and Kane,S.E. Differential sensitivities of trastuzumab (Herceptin(R))-resistant human breast cancer cells to phosphoinositide-3 kinase (PI-3K) and epidermal growth factor receptor (EGFR) kinase inhibitors, *Breast Cancer Res Treat.*, 91: 187-201, 2005.
- 9 Nagata,Y., Lan,K.H., Zhou,X., Tan,M., Esteva,F.J., Sahin,A.A., Klos,K.S., Li,P., Monia,B.P. and Nguyen,N.T. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients, *Cancer Cell*, 6: 117-127, 2004.
- 10 Hudziak,R.M., Lewis,G.D., Winget,M., Fendly,B.M., Shepart,H.M. and Ullrich,A. p185^{HER2} monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor, *Mol.Cell.Biol.*, 9: 1165-1172, 1989.
- 11 Yakes,F.M., Chinratanalab,W., Ritter,C.A., King,W., Seelig,S. and Arteaga,C.L. Herceptin-induced Inhibition of Phosphatidylinositol-3 Kinase and Akt Is Required for Antibody-mediated Effects on p27, Cyclin D1, and Antitumor Action, *Can.Res.*, 62: 4132-4141, 2002.
- 12 Le,X.F., Lammayot,A., Gold,D., Lu,Y., Mao,W., Chang,T., Patel,A., Mills,G.B. and Bast,R.C., Jr. Genes affecting the cell cycle, growth, maintenance, and drug sensitivity are preferentially regulated by anti-HER2 antibody through phosphatidylinositol 3-kinase-AKT signaling, *J Biol Chem.*, 280: 2092-2104, 2005.
- 13 Elstrom,R.L., Bauer,D.E., Buzzai,M., Karnauskas,R., Harris,M.H., Plas,D.R., Zhuang,H., Cinalli,R.M., Alavi,A., Rudin,C.M. and Thompson,C.B. Akt Stimulates Aerobic Glycolysis in Cancer Cells, *Can.Res.*, 64: 3892-3899, 2004.
- 14 Baldwin,S.A., Kan,O., Whetton,A.D., Martin,S., Fawcett,H.A.C., Flint,D.J. and Wilde,C.J. Regulation of the glucose transporter GLUT1 in mammalian cells, *Biochemical Society Transactions*, 22: 814-817, 1994.
- 15 Laughner,E., Taghavi,P., Chiles,K., Mahon,P.C. and Semenza,G.L. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression, *Mol Cell Biol*, 21: 3995-4004, 2001.
- 16 Liu,L. and Simon,M.C. Regulation of transcription and translation by hypoxia, *Cancer Biol Ther*, 3: 492-497, 2004.